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THE INDUCTION OF ALANINE-RICH PROTEINS DURING LOW TEMPERATURE ACCLIMATION OF *BRASSICA NAPUS* CV. CASCADE

BY

MEI (XIAOMEI) GENG

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

PLANT MOLECULAR BIOLOGY AND BIOTECHNOLOGY

DEPARTMENT OF PLANT SCIENCE

EDMONTON, ALBERTA

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DATE: Dec 25/97

ABSTRACT

The objective of this study was to determine if there was a class of alanine-rich proteins induced by low temperature that have a role in freezing tolerance. In winter *Brassica napus* cv. Cascade leaves, freezing tolerance was an inducible characteristic regulated by exposure to low temperature. Freezing tests showed that there were no changes in electrolyte leakage after one day of low temperature treatment, but acclimation was initiated within 4 days of low temperature exposure. Labeling of the fourth leaf of non-acclimated and acclimated seedlings *in vivo* with either [^{35}S]-methionine or [^{3}H]-alanine revealed two classes of polypeptides induced by low temperature. One consisted of low molecular weight alanine-rich proteins (molecular mass ranging between 6.5 kD and 14 kD). They were characterized as being induced by one day of low temperature treatment, labeled with [^{3}H]-alanine and not labeled with [^{35}S]-methionine. This is the first report of alanine-rich, methionine-poor polypeptides. We speculate that the low temperature-induced alanine-rich polypeptides in *Brassica* have a role either in adjusting metabolism during plants growth at low temperature or in developing freezing tolerance. The second class of polypeptides appeared on the fourth day of acclimation. Thus, the appearance of these polypeptides coincided with the onset of freezing tolerance. Northern blotting with an oligonucleotide coding for the alanine-rich region of bn28 gene (Orr et al., 1992), a low temperature induced gene from *Brassica napus*, showed that the bn28 transcript appeared on the first day of low temperature acclimation, remained throughout the experimental period and disappeared within 12 hours of deacclimation. The results from both *in vitro* translations and Northern blotting suggest that newly synthesized proteins during low temperature

acclimation were associated with specific gene expression which was regulated at the transcriptional level. The probe failed to detect any other transcripts, even at low stringency. Thus, the alanine-rich proteins identified in this study were encoded by several different genes containing alanine-rich motifs different from that form in antifreeze proteins.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CAP	Cold acclimated protein
COR	Cold regulated
CPM	Count per minute
DEPC	Diethlypyrocarbonate
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetate
Kb	Kilobase
kD	Kilodalton
LT50	Lethal temperature at 50% ion leakage
O.D.	Optical density
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethylsulfony fluoride
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid
Tris	Tris (hydroxymethyl) methylamine

Introduction

Temperature stress is a major factor that determines the distribution of plants in nature. In addition, temperature extremes limit the yield of crop plants in many agricultural regions of the world (Christiansen, 1982). Both high and low temperatures affect plant growth and development. In Canada, however, freezing rather than heat tends to be the major stress.

From the very beginning, research into the processes involved in freezing injury in plants has been directed primarily toward elucidation of the causes of cell death (Sakai and Larcher, 1987). Of the many hypotheses and theories that have been proposed to-date, none has proved entirely satisfactory. The main reason for this lies in the diversity of metabolic changes that accompany freezing injury. Ice formation and the ensuing injuries take different courses depending upon species, state of hardiness and the conditions of freezing. Therefore, no single, generally effective mechanism, can be said to be responsible for cell death and survival (Steponkus, 1981).

Plants differ in their ability to withstand freezing temperatures. For instance, many plant species develop freezing tolerance in response to low nonfreezing temperature. The woody perennials, birch and dogwood, offer an extreme example. Whereas nonacclimated trees are severely injured or killed at about -10°C, trees that are fully low temperature acclimated can survive temperatures of -196°C under experimental condition; in their natural environments, these trees are often exposed to, and survive, temperatures of -40°C to -50°C (Thomashow, 1990; Sakai and Larcher, 1987). The molecular basis for this response, termed low temperature acclimation, is poorly understood.

A number of biochemical changes occur in plants during low temperature acclimation. Common examples include alterations in

membrane phospholipid composition, appearance of new isozymes, increases in sugar and soluble protein content, and increases in levels of organic acids (Sakai and Larcher, 1987; Steponkus, 1984). Some of these changes presumably have important roles in freezing tolerance, whereas others may simply contribute to the overall fitness of plants for low-temperature survival (Thomashow, 1990). However, the precise role that each change has in low temperature acclimation remains uncertain.

The physical and biochemical changes that occur in plants during low temperature acclimation could be brought about by preexisting structures (for example, plasma membrane) and enzymes that undergo alterations in their properties at low temperature. In addition, changes in gene expression during low temperature acclimation have been established in a wide range of plants (Guy, 1990; Thomashow, 1990). Recently, direct evidence was obtained to show that low temperature induced the accumulation of specific mRNAs during acclimation. The appearance of novel transcripts during acclimation has been observed in alfalfa (Mohapatra et al., 1989), wheat (Lin et al., 1990; Honde et al., 1991), *Arabidopsis* (Hajela et al., 1990; Kurkela and Frank, 1990; Gilmour et al., 1992), and *Brassica* (Orr et al., 1992). Furthermore, DNA sequences corresponding to these cold-specific or low temperature regulated transcripts have also been isolated and characterized by differential screening of cDNA libraries constructed from these species.

Some of these cold-induced transcripts encode polypeptides containing stretches rich in alanine and glycine (Kurkela and Franck, 1990; Gilmour et al., 1992; Orr et al., 1992). Kurkela and Franck (1990) reported that a cold-regulated gene from *Arabidopsis* (Kin1) encoded an alanine-rich polypeptide (KIN 1 protein) with an amino acid sequence similar to that in group one fish

antifreeze proteins (AFPs). On this basis, they suggested that KIN 1 is an endogenous AFP in plants.

AFPs occur in certain arctic and antarctic fish in order to prevent serum freezing during periods of sub-zero ocean temperatures. These proteins lower the freezing temperature (i.e. temperature at which ice forms) without significantly affecting the melting temperature. This hysteretic effect is thought to be a kinetic consequence of the interaction of AFPs with ice (Burcham et al., 1986) and is non-colligative. The best characterized of these proteins was isolated from winter flounder, has a low molecular weight, is alanine-rich and alpha-helical (Yang et al., 1988). Similar proteins have been found in insects (Duman, 1979). Cutler et al., (1989) vacuum infiltrated AFP (winter flounder) into leaves of potato, canola (*Brassica napus*) and *Arabidopsis*. This resulted in significant depression of the spontaneous freezing temperature, relative to water infiltrated controls. In the case of canola, the freezing temperature decreased by an average of 1.8°C. Further, transfer of the flounder AFP gene into plants has been shown to improve freezing tolerance (Hightower et al., 1991). These results demonstrate that AFP has significant potential for improving the cold hardiness of plants.

Griffith and corworkers (1993) reported that winter rye (*Secale cereale* L.) leaves accumulate proteins in the apoplast during low temperature acclimation and that apoplastic extracts containing these proteins have the ability to modify ice. Partially purified apoplastic proteins obtained from winter rye leaves form ice crystals in solution that are hexagonal bipyramids and exhibit a thermal hysteresis of 0.3°C at a concentration of 60mg of protein/mL. This antifreeze activity was eliminated by treating the extracts with protease. This result suggests that AFPs are produced by winter rye. More recently, they demonstrated that a number of individual polypeptides isolated

from apoplastic extracts obtained from winter rye leaves have antifreeze activity. They also presented a comparison of these polypeptides with AFPs isolated from fish and insect, then concluded that AFPs in winter rye leaves appeared to be distinct from antifreezes produced by fish and insects.

Literature Review

Gene expression during low temperature acclimation

Isozyme composition: Enzyme changes are associated with the induction of freezing tolerance. These are reflected by the many biochemical changes plants undergo during low temperature acclimation. Although the enzyme changes may not be causally related to direct freezing injury, they are an integral component of the overall acclimation process (Johnson-Flanagan and Singh, 1988). Temperature can have a variety of effects on the structures and functions of enzymes. It affects the rate at which covalent bonds are made or broken during enzyme catalysis; it also affects the "weak" chemical bonds - hydrogen bonds, electrostatic interactions, hydrophobic interactions - that stabilize the higher orders of protein structure (secondary, tertiary, and quaternary structure) and enzyme-ligand interactions. For example, hydrogen bonds and electrostatic interactions, because they form exothermically, are stabilized by reductions in temperature. Hydrophobic interactions, which form endothermically, are favored by increases in temperature (Thomashow, 1990). Thus, enzyme structure and function can be modified by low temperature acclimation.

Different isozymes, better suited to low-temperature environments, might be produced during low temperature-acclimation. A number of examples of low temperature-acclimation-associated changes in various isozyme profiles have been documented (Sakai and Larcher, 1987). For example, Krasnnk et al., (1976) reported new isozymes of lactate dehydrogenase, glucose-6-phosphate dehydrogenase, and isocitrate dehydrogenase during low temperature acclimation in alfalfa, in addition to quantitative increases in a number of other isozymes. These changes were associated with increased cold tolerance. Whether the differences in isozyme profiles that were observed in most of these studies resulted from

posttranslational modifications of the enzyme or from changes in gene expression is not clear. However, Shomer-Ilah and Waised (1975), demonstrated that the cold-acclimation-associated Rubisco isozymes of cabbage were significantly different in amino acid composition between nonacclimated and acclimated plants. Thus, changes in gene expression were probably involved.

Protein content: Soluble proteins increase during the induction of freezing tolerance in many plants (Guy, 1990). Chen and Li (1980b) examined and analyzed frost-tolerant and frost-susceptible potato leaves and found that the linear regression of hardiness and soluble protein content had a correlation coefficient greater than 0.97.

Initially, it was not know whether the increase in soluble protein resulted from decreased utilization of proteins as a consequence of low growth rates or from increased synthesis. Siminovitch and co-workers (1968) addressed this problem by following protein synthesis with radioactive labeling. They reported increased incorporation during the period of acclimation. The finding that cycloheximide, an inhibitor of protein synthesis, can prevent the increased frost tolerance that normally occurs in cold treated wheat (Trunova, 1982) and potato (Chen et al.,1983) leaves is consistent with Siminovitch's report. Numerous reports have since clearly established that plants, when exposed to low temperature, synthesize new proteins (see references cited by Guy, 1990). However, unlike the heat-shock and anaerobic stress responses, wherein extensive changes in the patterns of protein synthesis occur (Kimpel et al. 1985; Sachs and Ho, 1986), the changes in protein synthesis during low temperature acclimation appear to be relatively modest. For, example, Guy and Haskell (1987) examined the

synthesis of polypeptides during low temperature acclimation in spinach by radiolabeling plant leaves *in vivo* with [^{35}S] methionine and analyzing the polypeptides by two-dimensional gel electrophoresis. They could resolve about 500 different polypeptides and found that most of the polypeptides synthesized by the control and low temperature-acclimated leaves were the same, but there were certain reproducible differences. Exposure of plants to 5°C resulted in the appearance of three new proteins (160, 117, and 85 kD); one protein dramatically increased, and nine polypeptides disappeared compared with nonacclimated plants. Other studies on *Arabidopsis* (Kurkela et al., 1988; Gilmour et al., 1988) documented low temperature-acclimation-associated changes in protein composition similar to those reported by Guy and Haskell (1987). Kurkela and Franck (1988) used *in vivo* radiolabeling and one-dimensional SDS-PAGE to compare the polypeptide profiles of nonacclimated and acclimated *Arabidopsis* and found that seven polypeptides dramatically increased in the acclimated plants; a few others were severely reduced. As with spinach, however, the overall polypeptide profiles of the acclimated and nonacclimated plants were very similar.

Guy and Haskell (1987) also showed that synthesis of the low-temperature-regulated three proteins (160, 117 and 85kD) closely paralleled freezing tolerance. That is, the appearance of these proteins generally coincided with the onset of freezing tolerance; their synthesis continued for as long as plants were kept at low temperature, and their synthesis declined quickly during deacclimation (induced by returning plants to nonacclimating temperature). For example, their time course studies indicated that freezing tolerance of spinach leaves increased after 24 hours at low temperature (5°C), and that maximum freezing tolerance occured in about 7 days (nonacclimated and acclimated leaves had LT50s of about -5°C and -10°C, respectively).

Returning the acclimated plants to warm temperatures (25°C) results in quick deacclimation; a loss in freezing tolerance was detected after 24 hours, and by 3 days, freezing tolerance was the same as for nonacclimated plants. Therefore, they concluded that during low temperature acclimation, the synthesis of these proteins was upregulated, while during deacclimation synthesis was downregulated.

The new proteins synthesized at low temperature may play a central role in freezing tolerance (Guy, 1990). *In vivo* radiolabeling studies in *Arabidopsis* demonstrated synthesis of two COR (cold regulated) proteins (160 and 47 kD) within 24 hours of exposing the plants to low temperature (Gilmour et al., 1988; Kurkela et al., 1988). The COR proteins continued to be synthesized for as long as the plants were kept at the acclimation temperature. Further, it was found that returning the plants to 25°C resulted in rapid loss of synthesis of these polypeptides. After three days, their synthesis was barely detectable. A similar correlation between COR protein synthesis and acclimation/deacclimation was obtained with alfalfa (Mohapatra *et al.*, 1987a, b).

RNA content: As early as 1970, Weiser proposed that low temperature acclimation of temperate woody perennials may require both transcriptional activation of a set of genes normally not expressed under non-acclimating conditions and the synthesis of new proteins (Weiser, 1970). In general, total RNA increases during the induction of freezing tolerance (Sarhan et al., 1985). Increased RNA content during acclimation may reflect a variety of changes in the protein synthesizing machinery ranging from overall increases in ribosomal (r)RNA and soluble (s)RNA (comprising messenger and transfer RNA), to a combination of increases and decreases of the RNA classes

(Johnson-Flanagan and Singh, 1988). A number of workers have reported increases in rRNA. For instance, Gusta et al. (1972) found that there was a 50% increase in the rate of rRNA synthesis in boxwood leaves during acclimation. Similarly, increases in rRNA were found to accompany freezing tolerance in potato (Oslund et al., 1972), and winter wheat (Paldi et al., 1982). sRNA also increased during low temperature acclimation of potato (Li et al., 1969) and mimosa epicotyles (Brown et al., 1972). RNA polymerase I was more active than RNA polymerase II during low temperature acclimation of winter wheat, suggesting more rapid synthesis of rRNA in comparison with mRNA (Sarhan and Chevrier, 1985).

The first direct evidence to support Weiser's (1970) hypothesis of altered gene expression during low temperature acclimation was the observation that newly translatable mRNAs appeared in spinach leaves exposed to 5°C (Guy et al., 1985). In their studies of spinach, *in vitro* translation assays showed that poly(A)$^+$ RNA from acclimated plants produced polypeptides that were not synthesized by poly (A)$^+$ RNA isolated from nonacclimated plants. It was also clear from the data that the overall *in vitro* translation patterns of poly(A)$^+$ RNA isolated from the acclimated and nonacclimated plants were similar.

Subsequent studies confirmed and extended this finding. Gilmour et al., (1988) and Thomashow et al., (1990) obtained evidence for increases in translatable mRNAs encoding polypeptides of 160, 47, 24, and 15 kD in low temperature acclimated *Arabidopsis*. These changes occurred rapidly, with increases for all of the mRNAs evident within 24 hours of exposing plants to low temperature. Mohapatra et al., (1987a) found increases in translatable mRNAs for seven polypeptides in low temperature-acclimated alfalfa, and Johnson-Flanagan and Singh (1987) found increases in mRNAs encoding

polypeptides of 20 kD and 17 kD in low temperature-acclimated *Brassica* suspension cultured cells.

Low temperature regulated genes: Direct evidence for increased levels of specific RNAs in low temperature acclimated plants has now been obtained by Northern blot analysis using isolated cDNA and genomic clones of cor (cold regulated) genes. Hajela et al. (1990) isolated four cDNA clones by constructing a cDNA library of poly(A)$^+$ RNA isolated from low temperature-acclimated *Arabidopsis* and screening it by differential hybridization to cDNA probes prepared against RNA isolated from acclimated and nonacclimated plants. Northern blot analysis of total RNA isolated from acclimated and nonacclimated plants indicated that four cor genes hybridized to transcripts of 1.4, 2.5, 0.7, and 0.6 Kb, respectively. The levels of these transcripts increased dramatically (10-fold or greater) in acclimated plants and returned to low levels in deacclimated plants. Time course studies indicated that the levels of all four cor transcripts increased markedly within 4 hours, continued to increase up to about 12 hours, and then remained at elevated levels for as long as the plants were kept at low temperatures (14 days). Returning the plants to warm temperatures resulted in rapid decreases in the levels of all four transcripts; within 4-8 hours, they fell to very low or undetectable levels. Nuclear run-on transcription and steady state assays indicated that the temperature-regulated accumulation of the three cor gene transcripts were controlled primarily at the posttranscriptional level, while accumulation of one cor gene transcript was regulated largely at the transcriptional level. Similarly, numerous other workers have demonstrated induction of low temperature regulated genes during low temperature acclimation in crops such as alfalfa (Mohapatra et al., 1988b, 1989 and Wolfraim et al., 1993), barley

(Cattivelli et al., 1990; Dunn et al., 1990), *Brassica* (Orr et al., 1992), *Maize* (Christie et al., 1991), wheat (Lin et al. 1990; Houde et al., 1992), tomato (Schaffer et al., 1988) and spinach (Neven L. G. et al., 1993).

Characterization of low temperature regulated genes and polypeptides

Correlation between low temperature regulated gene expression and freezing tolerance: Does low temperature-induced gene expression correlate with the degree of freezing tolerance obtained by cold acclimation? Mohapatra et al. (1989) isolated total RNA from acclimated and nonacclimated alfalfa plants and the level of cor gene expression was monitored using three alfalfa cor cDNA clones (pSM784, pSM2358 and pSM2201). Another cDNA clone (which was also from alfalfa) representing a gene (pSM355) that was expressed equally in acclimated and nonacclimated plants was used as a control. The alfalfa cultivars tested were Anik (*Medicago falcata*), Iroquois (*Medicago media*), Algonquin (*Medicago media*), and Trek (*Medicago sativa).* Freezing tolerance was assessed on the basis of LT50. The results indicated that a high, positive correlation between the level of the cold-acclimation-induced transcript accumulation and the increase in freezing tolerance obtained by these cultivars (for example, the cultivar Trek with an LT50 value of -9.7°C was the least freezing tolerant of the cultivars and showed the lowest level of expression cor genes. The cultivar Anik, on the other hand, was the most. freezing tolerant with an LT50 value of -14.6°C and showed the highest level of expression of cor genes. Moreover, cultivars Algonquin and Iroquois, which exhibit intermediate levels of freezing tolerance, showed intermediate levels of expression of these cor genes.). However, whether this correlation

will also be observed with other cor genes in other plants remains to be determined.

Biochemical properties of low temperature regulated polypeptides: Lin et al. (1990) found that at least four of the cor gene products of *Arabidopsis* share the unusual biochemical property of having "boiling solubility". That is, while most polypeptides are denatured and form a precipitate upon boiling, the major COR polypeptides remain soluble after such treatment. The full significance of this unusual biochemical property is not known. However, it is interesting to note that three families of polypeptides with properties that could potentially have roles in freezing tolerance have also been shown to be boiling soluble. One family consists of of low molecular weight antifreeze proteins from fish (Feeney and Yeh, 1978; DeVies, 1983), which are suggested to inhibit or retard ice formation by binding to growing ice crystals. These proteins are very hydrophilic and have some members that are boiling-stable (Thomashow, 1990). A second family, the dehydrins, accumulated in response to water stress and ABA treatment in barley and other grasses, are very hydrophilic, and do not precipitate upon boiling (Jacobsen et al., 1989). They are hypothesized to protect plant seedlings from dehydration stress during drought. The production of desiccation-protective proteins could potentially help plant cells tolerate the severe dehydration that occurs during a freeze-thaw cycle (Thomashow, 1990). Similarly, LEA (late embryogenesis abundant) proteins are also hydrophilic and have desiccation-protective effects. Lastly, cryoprotective proteins described by Heber and colleagues (Volger and Heber, 1975; Heber et al., 1979), which are produced in low temperature acclimated, but not non-acclimated spinach plants are more than 1000-times more effective in protecting thylakoid membranes (on a molar basis) against

freezing damage than low-molecular-weight cryoprotectants such as sucrose and glycerol. Biochemical characterization of these polypeptides has indicated that they have a high content of polar amino acids, are very hydrophilic, and do not precipitate on boiling.

Recently, Kurkela and Franck (1990) isolated a clone containing the cold inducible gene, kin1, from *Arabidopsis*. The kin1 gene encodes a protein of 66 amino acids with a predicted molecular weight of 6.5 kD and an isoeletric point of 7.2. The deduced protein is quite hydrophilic, with three small hydrophobic regions and a high amount of α-helical structure. Its amino acid composition is rather unusual: it contains 22.7% alanine, 13.6% glycine and 13.6% lysine. The polypeptide contains two Asp-Ala-Ala-Ala-Gly repeats. Kurkela and Franck (1990) compared the deduced KIN1 protein with several group one AFPs of arctic flounder and found that the amount of identical amino acids between KIN1 and one small AFP is 28%, and the amount of biochemically similar amino acids was 41%. Similarities of the same order were found with other flounder AFP sequence (Davies et al., 1984 and Lin et. al.,1981). All of these AFPs are classified in the same group and are highly homologous to each other. The KIN1 and these AFPs have two additional structural characteristics in common: they are all rich in nonpolar amino acids and can assume an α-helical structure. However, the comparison is based on the predicted amino acid sequence (Kurkela and Franck, 1990). Therefore, whether the KIN1 protein is functionally related to the AFPs remains uncertain.

The physical locations and possible functions of low temperature regulated polypeptides: Studies on the distribution of the COR polypeptides within various tissues of low temperature-acclimated plants, and their physical

location within plant cells, have been carried out in several laboratories. Neven et al., (1993) found an 85 kD spinach protein in all aerial tissues as well as in roots. It is responsive to low temperature acclimation and remains soluble upon boiling. Subcellular fractionation studies of acclimated tissue indicates that the 85 kD protein (sometime called CAP85kD-cold acclimated protein) is present in the cytosol and endoplasmic reticulum (ER). While the cDNA for CAP85 does not appear to have a signal sequence to target it to the ER, genomic DNA and protein sequencing suggested that there could be another CAP85 gene, which could account for a second subcellular site. DNA sequence analysis of COR15 from *Arabidopsis* indicates that the COR15 polypeptide has an N-terminal amino acid sequence that closely resembles a transit peptide that targets protein to the structural compartment of chloroplasts (Lin and Thomashow, 1990). Immunological studies indicate that COR15 is processed *in vivo* to a polypeptide of about 9 kD and appears to be located in chloroplasts as it can be detected in soluble protein extracts prepared from chloroplasts, purified on Percoll gradients. The processed protein (COR15m) is soluble, hydrophilic, and predicted to form an amphipathic α-helix. More recently, Lin and Thomashow (1992b) reported that COR15 has potent cryoprotective activity in an *in vitro* cryoprotective assay. Specifically, COR15 was very effective in protecting the cold-labile enzyme lactate dehydrogenase against freeze-inactivation; on a concentration basis, it was about 10^6 times more effective than sucrose and 10^2 to 10^3 times more effective than other proteins including bovine serum albumin. In winter rye, Griffith et al., (1994) reported that AFPs were extracted from the apoplast and may be associated with the cell wall, which is largely composed of carbohydrates. Thus, the rye AFPs might hydrogen bond to hydroxyl groups

present in the cell wall in much the same way that these proteins form hydrogen bonds with the ice crystal lattice.

Study objectives

Research to-date has suggested that there are antifreeze-type proteins in plants that are rich in alanine. Unfortunately, all evidence in support of plant AFPs is circumstantial as it is based on gene sequences. The overall objective of this study, therefore, is to determine whether alanine rich proteins are synthesized during low temperature acclimation in *Brassica napus*. If these proteins exist, their synthesis will be related to the attainment of freezing tolerance, and their boiling solubility will be assessed, as this could give a clue to the function of the proteins (As we mentioned in literature review, boiling solubility has been considered as an common biochemical property of cor gene products). Finally, to be able to definitively relate structure to function, homology between the alanine-rich proteins must exist.

The specific study objectives were as follows:

1. To test the hypothesis that endogenous alanine-rich proteins are induced by low temperature acclimation in *Brassica*.
2. To determine the relationship between freezing tolerance and the appearance of alanine-rich proteins. This requires analysis of proteins that are differentially labeled by methionine and alanine.
3. To examine the boiling solubility of polypeptides whose synthesis can be correlated with the induction of freezing tolerance.
4. To test the hypothesis that alanine-rich proteins contain a consensus sequence similar to that proposed to exist between KIN 1 and the type-one AFPs (This is the main argument in support of the antifreeze function proposed for alanine-rich proteins in plants).

Materials and Methods

Plant growth conditions and low temperature acclimation

Canola (*Brassica napus* cv. Cascade) plants were grown in sand:peat: soil (1:1:1) under greenhouse conditions at 20°C with augmented light (400W high pressure sodium vapor, Sylvania Lighting Products, Sylvania Canada Ltd., Edmonton, Canada) to provide a minimum irradiance of 200 µmol $m^{-2}s^{-1}$ for 16 hours per day. Plants were watered to the point of runoff three times per week.

When plants were at the 4 leaf stage (about 3 weeks, Figure III 1), some plants were moved to a cold room at a constant 4°C for low temperature acclimation (from 1 day up to 42 days). Non-acclimated plants were maintained at 20°C. Only the fourth leaves of acclimated and non-acclimated plants and growth at the same development stage were used for experimentation, as these have been shown to achieve the highest degree of freezing tolerance.

Evaluation of freezing tolerance

The fourth leaves of non-acclimated and acclimated plants were collected and washed with distilled water then blotted dry. One cm diameter cork borer was used to prepare leaf discs. Two discs were prepared per leaf. Freezing tolerance of leaves was determined by the method of Sukumaran and Weiser (1972). Excised leaf discs were placed 3 per plate in 60 x 15 mm disposable petri plates on moistened (with distilled water) 5.5 cm Whatman filter paper. The petri plates were placed in a programmable freezer at 0°C, the 0°C samples were removed and the temperature was lowered to -2.5°C during a one hour period. Following another one hour equilibration period set at -2.5°C, freezing was initiated by the addition of ice chips to each plate. After a 16-hour equilibration period, the -2.5°C samples were removed and the

temperature was lowered by 2.5°C/hour The samples were collected at 2.5°C intervals between -2.5°C and -10°C. Three plates were removed at each desired temperature (0, -2.5, -5.0, -7.5, and -10°C), and allowed to thaw at 4°C in the light for at least 12 hours, discs were placed in 50 ml deionized water and shaken overnight on room temperature. Cell damage was measured by leakage of electrolytes from the tissue into deionized water with CDM 83 conductivity meter (Radiometer). The samples were then boiled for 5 minutes in a microwave, the volume adjusted to 50ml, shaken for one hour at room temperature, and the conductivity measured. Conductivity of the leachate was expressed as the percentage of that obtained after boiling. The temperature at 50% ion leakage was taken to be the lethal temperature (LT50). The freezing tolerance was described by the the LT50 value. The experiment was replicated three times.

Total RNA extraction

Total RNA from non-acclimated and acclimated fourth leaves were prepared by a modified phenol/chloroform method (Current Protocols in Molecular Biology, 1990). Leaves were detached, rinsed with distilled water, blotted dry, frozen in liquid N_2 and stored at -80°C. The frozen tissue was ground to a fine powder in liquid N_2. Guanidinium isothiocyanate buffer (50% [w/v] guanidinium isothiocyanate, 2% [w/v] sarcosyl, 50mM Tris-HCl [pH 8.0], 1% [v/v] 2-mercaptoethanol) containing sodium acetate (final concentration of 0.2M), SDS (final concentration 2% [w/v]), an equal volume of phenol and 1/2 volume of chloroform: isoamyl alcohol (24:1) was added in the ratio of 5ml/g fresh weight and was vortexed vigorously, then incubated on ice for 15 minutes. Following centrifugation at 10,000 x g for 20 minutes at 4°C, the aqueous phase was removed, and extracted twice (once with an equal

volume of phenol:chloroform (2:1); once with an equal volume of chloroform only). The RNA was precipitated from the aqueous phase with an equal volume of ice-cold isopropanol at -20°C for at least one hour. The RNA pellet was collected by centrifugation at 10,000 x g for 20 minutes at 4°C, and redissolved in TE buffer (10mM Tris-HCl and 1mM EDTA pH 8.0) and reextracted with an equal volume of phenol:chloroform (2:1), followed by an equal volume of chloroform until a clear interface was obtained. The RNA was precipitated again with one tenth volume of 3M sodium acetate (pH 5.2) and 2.5 volume of cold ethanol at -20°C overnight, then precipitated by centrifugation at 10,000 x g for 30 min at 4°C. The pellet was washed with 75% ethanol, then dissolved in DEPC-treated water (Current Protocols in Molecular Biology, 1990). RNA concentration was determined by absorbance at 260 nm with an O.D of 1 taken as 40 µg/ml. The 260/280 ratio was within 1.8 to 2.0.

In vivo radiolabeling and protein extraction

Non-acclimated and acclimated plants were *in vivo* radio labeled with [^{35}S] methionine (1480 curies/mmol, Amersham) and [^{3}H] alanine (58 curies/mmol, Amersham). L-[^{35}S] methionine and L-[^{3}H] alanine were diluted to a concentration of 1 µCi/µl with sterile distilled water containing 0.01% v/v Triton X-100. On the day prior to labeling, the plants were trimmed to a single leaf. The radiolabeling solution (total volume of 8.5 µl) was applied to the underside (abaxial side) of the leaf. The plants were then incubated at 20°C for 4 hours or 4°C for 12 hours (In our preliminary experiments, we found that the incorporation of [^{35}S]-methionine into non-acclimated leaves was three times faster than that into acclimated leaves. Thus, in all of our *in vivo* labeling experiments, we labeled non-acclimated leaves for 4 hours and

acclimated leaves for 12 hours.). Thereafter, the leaves were harvested, rinsed with distilled water, then frozen with liquid N_2 and kept at -80°C.

Frozen leaves were ground with liquid N_2 and sand in a cold mortar and homogenized in 0.4 ml of Laemmli (Laemmli, 1970) sample buffer (60 mM Tris-HCl [pH 6.8], 10% [w/v] glycerol, 2% [w/v] SDS, 5%[v/v] 2-mercaptoethanol) with 0.01% PMSF. The homogenate was centrifuged for 10 minutes at 13,000 x g at 4°C to pellet cell debris and the supernatant was used for protein analysis. Two µl of the sample was spotted onto a Whatman GF/C filter disc (in duplicate). The filter discs were dried, washed twice with 10% [v/v] TCA, once with 5% [v/v] TCA, and once with 95% ethanol, and then dried. Incorporation of radiolabel was determined by liquid scintillation counting. Protein was quantitated by the Bradford method with BSA as a standard (Bradford, 1976).

SDS-PAGE

Radiolabeled proteins were separated by SDS-PAGE (Mini-PROTEIN ® II Electrophoresis Cell, BIO-RAD). Equal amounts of TCA insoluble radioactivity (62,500 dpm for [^{35}S]-methionine labeling and 8,350 dpm for [^{3}H]-alanine labeling) were loaded on 15% polyacrylamide gel containing 0.1% [v/v] SDS. The gels and buffer solutions were prepared as described by Laemmli (Laemmli, 1970). After electrophoresis at 150 volts for 1.5 hours at 20°C, the gels were stained with Coomassie blue (0.1 % [v/v] Coomassie blue R-250 in 40% [v/v] methanol and 10% [v/v] acetic acid) for one hour, then destained with 40% [v/v] methanol, 10% [v/v] acetic acid overnight. Then the gels were soaked in En^{3}hance (New England Nuclear) for one hour and 5%[v/v] glycerol for 30 minutes, and dried in a model 583 gel dryer (Bio-Rad Mississauga Ont.) for two hours. The dried gels were exposed to x-ray film for

3 to 4 days at -80°C for [^{35}S]-methionine labeled products and for 4 to 6 weeks at -80°C for [^{3}H]-alanine labeled proteins. FUJI RX film was used for all the fluorography.

In vitro translations of RNA and preparation of boiling soluble polypeptides

The rabbit reticulocyte lysate system (Amersham) was used in all *in vitro* translation experiments. The protocol described by the manufacturer was modified as follows. To get efficient translation, six different amounts (2, 5, 7.5, 15 , 25 and 50μg) of total RNAs and four different incubation times (30, 45, 60 and 90 minutes) were tested. To determine the optimal RNA concentration, the translation solutions were incubated at 33°C for 60 minutes. For the incubation time course, 25 μg of RNA was translated at 33°C for varying time (30, 45, 60 and 90 minutes). Following the reaction, 1 μl samples were spotted onto duplicate Whatman GF/C filter discs. The discs were dried, washed twice with 10% [v/v] TCA, once with 5% [v/v] TCA, and once with 95% ethanol, and then incorporation was determined by liquid scintillation counting.

The incorporation results showed that 25 μg total RNA and a one hour incubation time was optimum for our *in vitro* translations. The [^{35}S]-methionine labeled *in vitro* translation products were divided in half. An equal volume of 2 x Laemmli sample buffer (Laemmli, 1970) was added to one half and an equal volume of 2 x 50mM Tris-HCl (pH 7.0) was added to the other half. The samples in Tris-HCl were boiled for 10 minutes, and insoluble material removed by centrifugation in an Eppendorf microfuge (full speed for 15 minutes). The supernatant containing boiling soluble polypeptides was collected and added into an equal volume of 2 x Laemmli sample buffer as the

boiling soluble samples. For total protein samples (without boiling), 1 µl of each sample was used to calculate the incorporation of radiolabel (the method was the same as that used in *in vivo* labeling). An equal amount of TCA insoluble radioactivity (43,750 dpm) was then loaded onto a 12% [v/v] SDS-PAGE gel. For boiling soluble samples, double volumes of each total protein sample were loaded onto a 12% [v/v] SDS-PAGE gel. For the [^{14}C]-alanine samples, all *in vitro* translation products were loaded as the specific radioactivity was very low. The gel's preparation, electrophoresis, staining and fluorography were as described previously for *in vivo* labeled samples.

Northern blotting

Twenty µg of total RNAs from each sample were denatured and separated on a 1.2% [v/v] agarose gel containing 2.2M formaldehyde and 40 mM MOPS (pH 7.0), (Morpholinopropanesulfonic acid with 10 mM sodium acetate, and 1mM EDTA) (Current Protocols in Molecular Biology, 1990). Five µg of RNA molecular weight Marker III (Boehringer) was used as molecular weight standard. The size fractionated RNAs were transferred to ZETA-PROBE blotting membrane (Bio-Rad, Mississauga Ont.) by blotting with 10 x SSC (3M sodium chloride, 0.3 M sodium citrate) overnight by capillary action. The membrane was prehybridized in prehybridization buffer containing 5 x SSC, 20 mM NaH_2PO_4 (pH 7.0), 7% [v/v] SDS, 10 x Denhardt's (1% [w/v] Ficoll, 1% [w/v] polyvinylpyrrolidone and 1% [w/v] BSA) at 50°C for 5 hours.

A 27-base oligonucleotide (5'-CGCAGACGCTCCAGCTGCGGTAGC AGC-3'), complementary to the alanine-rich region of a low temperature induced gene bn28 (Orr et al., 1992) (Figure 2) was synthesized (Department of Microbiology, University of Alberta.)) and used as a probe to screen for transcripts in non-acclimated and acclimated Cascade leaves. Comparison to

the GenBank sequence data revealed significant homology between this region, two low temperature induced genes from *Arabidopsis* (Gilmour et al., 1992; Kurkela and Franck, 1990) and one group of antifreeze proteins from fish winter flounder (Hew et al., 1989). The alignment of the nucleotides and putative amino acid sequences are presented in Table 1 and Table 2.

The oligonucleotide was labeled with [γ-^{32}P] ATP at the '5 end by T4 polynucleotide kinase (Maniatis et al., 1982). The labeling reaction contained the following: 50 pmol DNA, 50 pmol [γ-^{32}P] ATP, 10ml 10 x kinase buffer 1, and 20 units of polynucleotide kinase in a final volume of 50 µl. The reaction was incubated at 37°C for 40 minutes, then diluted 2-fold in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0), and applied to a small column (1cm x 10cm) of Sephadex G-50 which had been equilibrated with TE (pH 8.0) to remove the unincorporated label. The labeled oligonucleotide was then used directly in the hybridization experiment.

After hybridization overnight at 50°C in the prehybridization buffer plus probe, the membrane was washed twice at 50°C for 30 minutes in 3 x SSC and, 10 x Denhardt's, 5% [v/v] SDS and 25mM NaH_2PO_4, and once at 50°C for 30 minutes in 1 x SSC, 1% [v/v] SDS. The moistened membrane was placed in a sealable plastic bag. Autoradiography was done using FUJI RX film at -80°C.

Figure 1. Winter *Brassica napus* cv. Cascade plant at four leaf stage

Figure 2. Nucleotide and deduced amino acid sequence of cDNA (bn28) from *Brassica napus* cv Jet Neuf (Orr et al. 1992). Underline indicates the oligonucleotide region.

```
1   AAAAAAACACAACAACTCAATAAATAAACAAATGGCAGACAACAAGCAGAGCTTCCAA
                                    M  A  D  N  K  Q  S  F  Q        9

61  GCCGGTCAAGCCTCTGGTCGTGCTGAGGAGAAGGGTAATGTGCTGATGGACAAGGTCAAG
      A  G  Q  A  S  G  R  A  E  E  K  G  N  V  L  M  D  K  V  K    29

121 GATGCTGCTACCGCAGCTGGAGCGTCTGCGCAAACCGCGGGACAGAAGATAACGGAGGCG
      D  A  A  T  A  A  G  A  S  A  Q  T  A  G  Q  K  I  T  E  A    49

181 GCAGGGGGAGCCGTTAATCTCGTGAAGGAGAAGACCGGCATGAACAAGTAGCCCCATTGG
      A  G  G  A  V  N  L  V  K  E  K  T  G  M  N  K                65

241 AAATAAAATTGGGAGTTATAGTTTCCCTTTTTAATGTTAATCGTTGTGGTTTTAAATAAA

361 AGTAATGTTTTCAACCTTTAGGTGTATGTCTTGATGTTTATGTACTTAGTTCCCCCTTAA
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Table 1. Alignment of the nucleotide sequences of alanine-rich regions of a number of low temperature induced genes

Brassica	bn 28	GCT GCT ACC GCA GCT GGA GCG TCT GCG	cold-induced
Arabidopsis	cor 6.6	GCT GCT GCT GCA GCT GGA GCT TCC GCG	cold-induced
Arabidopsis	kin1	GCT GCA GCT GGT GCT GGA GCT GGA GCA	cold-induced
Fish (Winter Flounder)	AFP 1	GCC GCC GCT GCA GCC GCC GCC AAC GCC	cold-induced

Table 2. Alignment of the deduced amino acid sequence of alanine-rich regions of a number of low temperature induced genes

Brassica	BN28	AATAAGASA	cold-induced	65AA
Arabidopsis	COR6.6	AAAAAGASA	cold-induced	66AA
Arabidopsis	KIN1	AAAGAGAGA	cold-induced	66AA
Fish (Winter Flounder)	AFP1	AAAAAAANA	cold-induced	82AA

Results

Induction of freezing tolerance during low temperature acclimation

The ability of winter *Brassica napus* cv. Cascade to cold acclimate was assessed by determining the freezing tolerance of the 4th leaves from non-acclimated (maintained at 20°C) and low temperature (4°C) acclimated plants. Electrolyte leakage is shown in Figure 3. The figure shows that there was little or no acclimation after one day at low temperature. However, the electrolyte leakage from four day acclimated leaves was significantly lower. The LT50s of acclimated leaves dropped from -2.5°C in control plants to -5.5°C after 4 days of acclimation. This indicates some acclimation within 4 days. The data (Table 3.) also shows that freezing tolerance was an inducible character in *Brassica napus* cv..Cascade, maximum tolerance can be achieved (-16°C) after 6 weeks of acclimation.

In vivo radiolabeling

Figure 4 shows the result from [^{35}S]-methionine *in vivo* labeling experiments. The protein pattern from low temperature treated leaves was similar to that of non-acclimated leaves. Little or no suppression of protein synthesis was found during the initial four day of low temperature acclimation. Most polypeptides synthesized under non-acclimating condition continued to be synthesized after transfer of plants to low temperature. In addition, some polypeptides were induced by low temperature For example, two polypeptides (21 kD and 37 kD) were induced by one day of low temperature treatment. Several proteins ranging between 66 kD and 130 kD were induced by 4 days of low temperature acclimation. No change was found in the low molecular weight polypeptides (range between 6.5 kD and 14 kD). Figure 5 shows the result of [^{3}H] alanine *in vivo* labeling. The labeled polypeptide pattern of low temperature treated leaves was different from

non-acclimated leaves. Two polypeptides (66 kD and 40 kD) disappeared after one day of low temperature acclimation. The synthesis of a 26 kD polypeptide was suppressed after 4 days of low temperature. However, a few polypeptides were induced by low temperature. A 43 kD polypeptides was induced transiently by one day of acclimation. Three low molecular weight polypeptides (13 kD, 8.5 kD and 6.5 kD) increased from the first day of low temperature acclimation, and were then maintained throughout the four day experimental time. It is interesting to note that some large molecular weight polypeptides were suppressed and low molecular polypeptides were induced during the low temperature acclimation period. By comparing the [^{35}S]-methionine labeled protein pattern with [^{3}H]-alanine labeled protein pattern, we found that most low temperature induced large molecular polypeptides (range between 66 kD - 130 kD) were detected in [^{35}S]-methionine labeled protein profile. and low molecular weight polypeptides (range between 6.5 kD and 14 kD) induced by low temperature were only identified in [^{3}H]-alanine labeled protein profile. Therefore, these low molecular polypeptides were alanine-rich, methionine poor polypeptides.

Difference in *in vitro* translation efficiency

Difference in the efficiency of incorporation in *in vitro* translations were noted between samples (Figure 6). The incorporation rate dropped when RNA was extracted from plants that had been moved from non-acclimating to acclimating conditions, and increased as the acclimating time increased. The efficiency of incorporation was not different between the non-acclimated and 4 day acclimated sample.

In vitro translations

We investigated changes in the pattern of polypeptides translated *in vitro*. Figure 7 shows a fluorograph of [^{35}S]-methionine labeled products from the *in vitro* translation of total RNA of non-acclimated and low temperature acclimated Cascade leaves. During the first two days of low temperature acclimation, transcription of mRNA coding for some larger molecular weight polypeptides was suppressed. However, they could be detected again following 4 days of acclimation (including a 39 kD polypeptide). We also observed a polypeptide synthesized in control plants that disappeared during the 4 day low temperature acclimation period (left, open arrow). It was more easily seen in the fluorograph of *in vitro* translated boiling soluble proteins (Figure 8). As anticipated, the boiling treatment resulted in precipitation of the majority of *in vitro* translation products from either the control or acclimated plants. However, some polypeptides in the non-acclimated leaves were found to be boiling soluble (open triangles, left side). Of these, one polypeptide (approximate molecular weight 20 kD) continued to appear during acclimation, while polypeptides of 31 kD and 51 kD apparent molecular weight were suppressed by the first day of acclimation and present again at 2 day of acclimation. mRNAs coding for three boiling soluble polypeptides of 115 kD, 47 kD and 39 kD apparent molecular weight (solid triangles, right side) were noted only during acclimation. Because synthesis of alanine-rich polypeptides did not appear to correlate with an increase in freezing tolerance, their stability following boiling was not determined.

Figure 9 shows the results of *in vitro* translations of total RNA from non-acclimated and low temperature acclimated Cascade leaves labeled with [^{14}C]-alanine. As mentioned in the Materials and Methods section, the entire translation reaction mixture was loaded for each treatment. The efficiency of

translation paralleled the [^{35}S]-methionine labeling results. The initial efficiency of *in vitro* translation decreased dramatically during the first day of acclimation, then increased as the exposure to low temperature increased. Since the sample load was not based on equal amounts of TCA insoluble radioactivity, it is difficult to tell which polypeptides disappeared during acclimation. However, it appears that two polypeptides were synthesized only during low temperature acclimation (approximate molecular weight 47 kD and 28 kD).

Northern blot analysis

Figure 10 shows the hybridization of total RNA from non-acclimated and low temperature acclimated Cascade leaves with the oligonucleotide complementary to a low temperature-induced gene (bn28) alanine-rich region (Figure 2). There was no hybridization between the oligonucleotide and any transcript other than the 0.45 Kb transcript expected to be bn28. No expression was detected from non-acclimated leaves (N). The transcript appeared on day one of low temperature acclimation (A1) and remained throughout the 42 days acclimation experimental period. It disappeared after a 12 hour deacclimation period (left side numbers indicate RNA molecular weight).

Figure 3. Effect of low temperature acclimation on freezing tolerance of *Brassica napus* (cv. Cascade) 4th leaves. Electrolyte leakage expressed as percent total electrolytes in tissue. Each value is a mean of three replicates and vertical bars indicate the standard error of the mean.

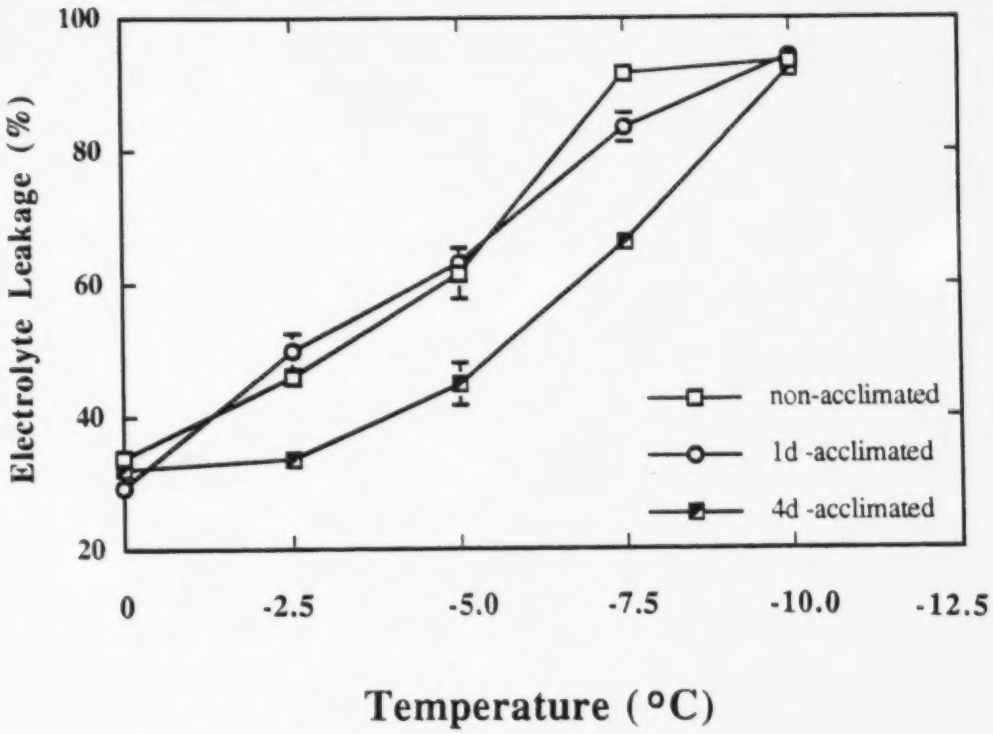

100
80
60
40
20
Electrolyte Leakage (%)
0
-2.5
-5.0
-7.5
-10.0
-12.5
non-acclimated
1d -acclimated
4d -acclimated
Temperature (°C)

Table 3. Freezing tolerance of *Brassica napus* cv. Cascade 4th leaves acclimated at 4°C for up to 42 days.

Time at 4°C	LT 50[a]
0d	-2.5°C ± 0.67[b]
4d	-5.5°C ± 0.37
14d	-11.1°C ± 0.68
28d	-12.7°C ± 0.50
42d	-16.0°C ± 0.38

a Each value is a mean of 3 replicates ± SE.

b The temperature at 50% ion leakage was taken to be the lethal temperature (LT50). The freezing tolerance was described by the LT50 value.

Figure 4. Fluorograph of *in vivo* [^{35}S]-methionine labeled polypeptides from non-acclimated (N) and acclimated 1 day and 4 day (A1 and A4) Cascade 4th leaves separated on 15% SDS-PAGE 62,500 dpm were loaded in each lane. Numbers with arrows indicate approximate molecular mass in kD of the polypeptides induced by low temperature. Numbers with bracket indicate the range of the polypeptides induced by low temperature.

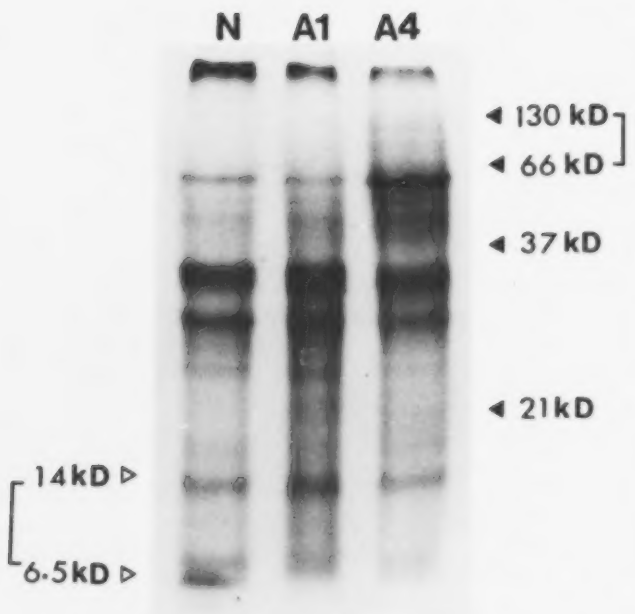

N
A1
A4
130 kD
66 kD
37 kD
21 kD
14kD
6.5kD

Figure 5. Fluorograph of *in vivo* [^{3}H]-alanine labeled polypeptides from non-acclimated (N) and acclimated 1 day and 4 day (A1 and A4) Cascade 4th leaves separated in 15% SDS-PAGE 8,350 dpm were loaded in each lane. Numbers with arrows indicate approximate molecular mass in kD of the polypeptides induced by low temperature. Numbers with bracket indicate the range of molecular mass in kD of the polypeptides induced by low temperature.

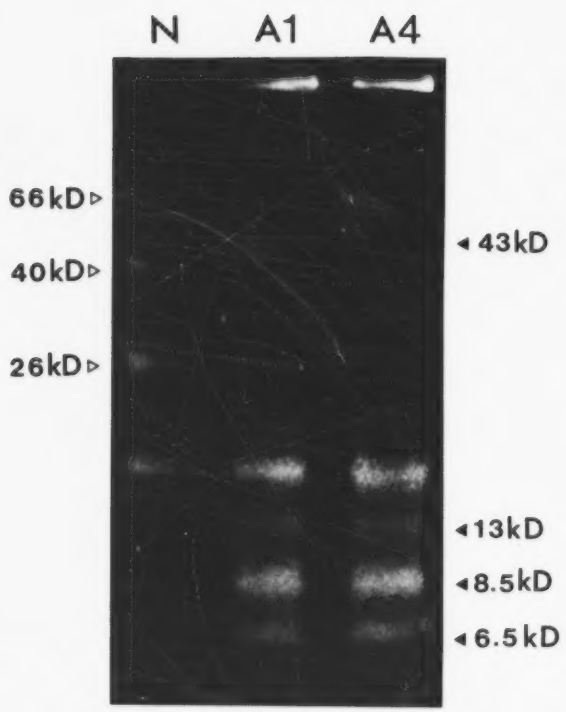

N
A1
A4
66kD
40kD
26kD
43kD
13kD
8.5kD
6.5kD

Figure 6. Low temperature induced changes in the efficiency of *in vitro* translations (0: without exogenous RNA). The same amount of total RNAs (25μg) for each of the samples (N: non-acclimation, A1: 1 day acclimated, A2: 2 day acclimated, A4: 4 day acclimated) were translated *in vitro* with [^{35}S]-methionine for 60 minutes. Each value is a mean of three replicates and vertical bars indicate the standard error of the mean.

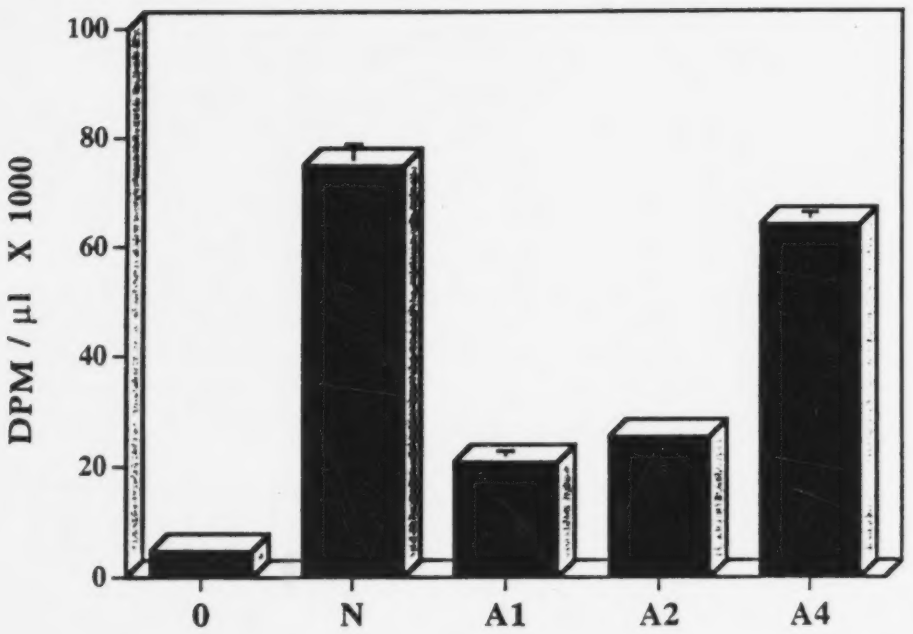

100
80
60
40
20
0
DPM / µl X 1000
0
N
A1
A2
A4
Sample time

Figure 7. Fluorograph of *in vitro* translation products labeled with [^{35}S]-methionine from total RNA of non-acclimated (N) and acclimated 1 day, 2 day and 4 day (A1, A2, A4) Cascade 4th leaves. (0: without exogenous RNA). Translation products were separated in 12% SDS-PAGE 62,500 dpm were loaded in each lane. Number with solid arrow indicates approximate molecular mass in kD of the polypeptide induced by low temperature. Number with open arrow indicates approximate molecular mass in kD of the polypeptide suppressed during low temperature.

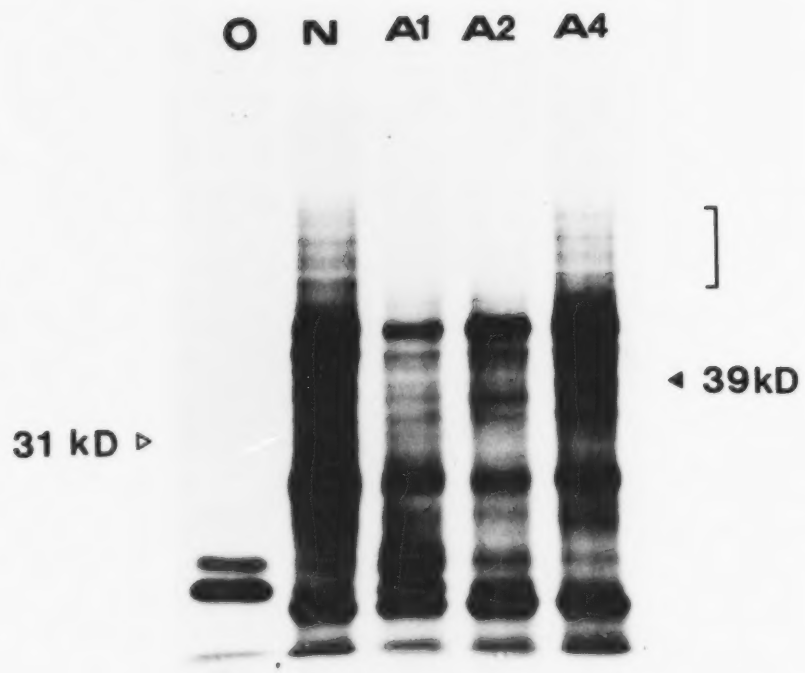

O
N
A1
A2
A4
39kD
31 kD

Figure 8. Fluorograph of boiling soluble products from *in vitro* translations labeled with [^{35}S]-methionine. Total RNA of non-acclimated (N) and acclimated 1 day, 2 day and 4 day (A1, A2, A4) Cascade 4th leaves (0: without exogenous RNA) was translated. The boiling soluble proteins were separated in 12% SDS-PAGE. Numbers with solid triangles indicate approximate molecular mass in kD of polypeptides induced by low temperature. Numbers with open triangles indicate approximate molecular mass in kD of polypeptides translated in both non-acclimated and acclimated plants.

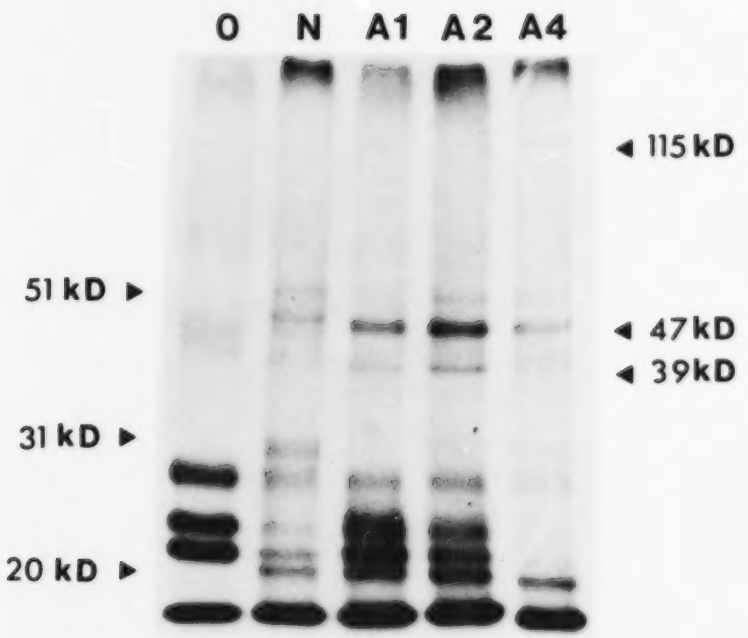

0
N
A1
A2
A4
115 kD
51 kD
47 kD
39 kD
31 kD
20 kD

Figure 9. Fluorograph of *in vitro* translation products labeled with [^{14}C]-alanine from total RNA of non-acclimated (N) and acclimated 1 day, 2 day and 4 day (A1, A2 and A4) Cascade 4th leaves. Translation products were separated in 12% SDS-PAGE. Numbers with arrows indicate approximate molecular mass in kD of polypeptides induced by low temperature. Total volum of translation products (25µl) was loaded.

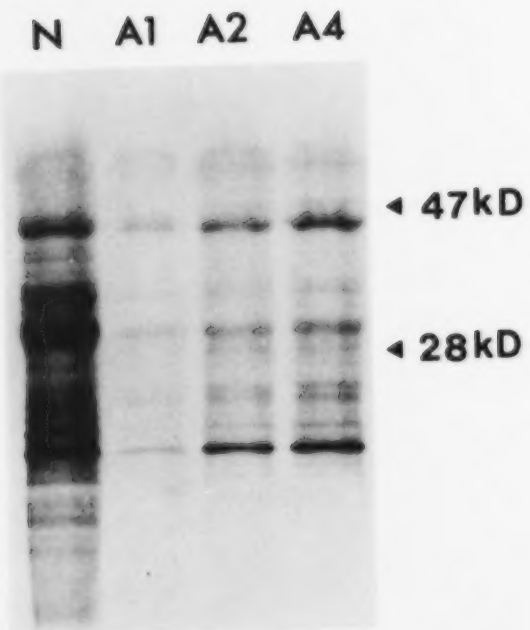
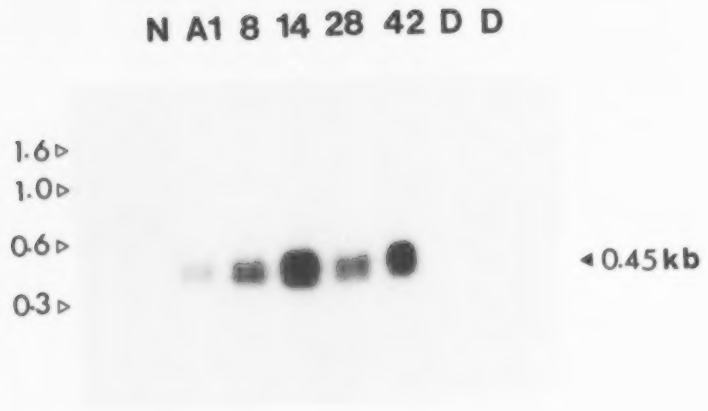

N
A1
A2
A4
47kD
28kD

Figure 10. Northern blot analysis of gene expression for the alanine-rich region of bn28 in Cascade. Total RNA was isolated from Cascade 4th leaves non-acclimated (N), acclimated 1 day, 8 day, 14 day, 28 day and 42 day (A1, A8, A14, A28 and A42) and deacclimated 12 hour and 24 hour (D) . The size of transcript is indicated in kilobases (kb) on the right. RNA molecular marker is indicated in the left.


N A1 8 14 28 42 D D
1.6
1.0
0.6
0.3
0.45kb

Discussion

In winter *Brassica napus* cv. Cascade, freezing tolerance was an inducible characteristic regulated by exposure to low temperature. Freezing tests show there are no changes in electrolyte leakage after one day of low temperature, but acclimation is initiated within 4 days of low temperature exposure. During this period of time, plant cells undergo physiological and metabolic changes to adjust the plant's metabolism to low temperature growth, and also to develop freezing tolerance (Antikainen et al., 1993).

Numerous reports have now clearly established that plants, when exposed to low temperature, synthesize new proteins, in addition to many of the proteins synthesized under non-acclimating conditions (Gilmour et al., 1988; Guy et al., 1985, 1987; Johnson-Flanagan et al., 1987; Kurkela et al., 1988; Mohapatra et al., 1987, 1988; Perras et al., 1989; Robertson et al., 1987). Our results are in agreement with the observation of others (Guy et al., 1987; Gilmour et al., 1988; Mohapatra et al., 1988). Therefore, low temperature stress is different from many other environmental stresses. For example, heat-shock and anaerobic stress result in extensive suppression of protein synthesis (Kimpel et al. 1985; Sachs et al., 1986).

Nearly all studies of protein synthesis during low temperature acclimation use [^{35}S]-methionine as a radiolabel. As we were interested in studying alanine-rich proteins, which may or may not contain methionine, we labeled with either [^{35}S]-methionine or [^{3}H]-alanine. Similarly, Monroy et al. (1992) by labeling with [^{3}H]-glycine studied expression of glycine-rich protein during low temperature acclimation in alfalfa.

By comparing [^{3}H]-alanine and [^{35}S]-methionine labeled protein profiles, we found that two classes of polypeptides were induced during low temperature acclimation. One is a group of low molecular weight alanine-rich proteins (Figure 5). They were induced by one day of low temperature

exposure. This is the first report identifying alanine-rich, methionine-poor polypeptides in plants. The second class of polypeptides appeared on the fourth day of acclimation and most of them appeared in [^{35}S]-methionine labeled profile. The appearance of these polypeptides coincided with the onset of freezing tolerance.

Three low molecular weight alanine-rich proteins were in the range of 6.5 kD to 14 kD. One of these (6.5 kD) is very close in size to the BN28 protein, which was predicted to be 6.5 kD from the cDNA sequence isolated and characterized from *Brassica napus* cv.Jet Neuf (Orr et al., 1992). BN28 is predicted to be an alanine-rich, methionine poor polypeptide. Whether the alanine-rich protein detected in the present study is BN28 needs to be verified by Western blotting.

The role of the low molecular weight alanine-rich polypeptides is unknown. They may be associated with the development of freezing tolerance or a response to adjust the plant's metabolism to low temperature. It is interesting to note that they were induced by a one day low temperature treatment whereas freezing tolerance increased at day 4. The effect of deacclimating temperature on the alanine-labeled polypeptides in the present study is not known. Further, Boothe and Johnson-Flanagan (unpublished) observed a loss of BN28 polypeptide within one week of deacclimating growth conditions that was not accompanied by a decline in freezing tolerance as measured by electrolyte leakage (transfered of acclimated plants to room temperature for two and seven days led to LT50 values of -13°C and -10°C respectively). Boothe et al. also (unpublished results) showed that the BN28 polypeptide is induced by low temperature in a spring cultivar of *Brassica* (Westar) that can also be acclimated to LT50 -10°C from -2°C. These results suggest that the low molecular weight alanine-rich proteins may have

a role in both adjusting the plant's metabolism to growth at low temperature and developing freezing tolerance. However, Danyluk and Sarhan (1990) suggested that the proteins encoded by mRNAs that were expressed only in freezing tolerant cultivars of wheat were responsible for the increase of freezing tolerance, and that other cold-inducible mRNAs that code for polypeptides required for cellular adjustment during growth at low temperature were present in both spring and winter cultivars and were not associated with the development of freezing tolerance.

The appearance of the second class of low temperature induced polypeptides coincides with the onset of freezing tolerance; however, the accumulation or decline of these polypeptides with increases or decreases in freezing tolerance have not been established. Other studies (Neven et. al. 1993) have attempted to correlate the presence of a specific polypeptide (CAP85-Cold Acclimation Protein, molecular weight 85 kD) with freezing tolerance. When CAP85 levels were high, there was a noticeable increase in freezing tolerance over control plants. Once plants were returned to non-acclimation conditions, the mRNA returned to control levels and freezing tolerance was lost. There was a considerable lag period between the decline in the mRNA and that of the protein. They suggested that slow protein degradation may provide protection during recurring periods of frost interspersed with short periods of warm temperature.

Lin and coworkers (1990) identified several cold-induced boiling-soluble polypeptides in *Arabidopsis* and wheat. They suggested that the boiling-soluble COR-polypeptides have a fundamental role in freezing tolerance and that the boiling-soluble nature of the polypeptides reflects their function. As previously indicated, three families of polypeptides with properties that could potentially have roles in freezing tolerance have been

shown to be boiling soluble (Thomashow 1990). We also used the boiling solubility to charactize *in vitro* translation products. We found that boiling-soluble polypeptides were not unique to acclimated leaves, as three boiling-soluble polypeptides (51 kD, 31 kD and 20 kD) were found in non-acclimated samples. Of these, one polypeptide (20 kD) continued to be present during acclimation while two others (51 kD and 31 kD) were suppressed by the first day of low temperature treatment and were present again by the second day of acclimation. In addition, three newly synthesized boiling-soluble polypeptides (39 kD, 47 kD and 115 kD) were induced by low temperature acclimation. One of these, a polypeptide of 115 kD apparent molecular weight, was detected on the fourth day of acclimation. This polypeptide was also observed in *in vivo* labeling experiments with methionine. These results suggest that the characteristic of boiling solubility may not be as closely linked to physiological function as suggested by Lin et al. (1990).

Our results from *in vitro* translation experiments showed that total translation rates dramatically decreased on the first day of exposure of plants to acclimating temperature (Figure 6). As acclimation time increased, there appeared to be a recovery by 4 days of acclimation, as the incorporation had almost reached the level of the control. Danyluk and Sarhan (1990) also found this phenomenon in wheat and suggested that it was possible some of the mRNA associated with freezing tolerance were not translated efficiently at the beginning of the acclimation period. Thus, proteins resulting from their translation would not be present in sufficient quantity needed for the development of freezing tolerance.

Alternatively, this decrease in translational rates could reflect alteration of the mRNA/rRNA ratio since we used equal amounts of total RNA. In wheat, Antikainen et al. (1993) observed that cold treatment caused an

increase in the polysomal level but translational efficiency *in vivo* was not stimulated. They suggested that the need for extra rRNA and polysomes is increased as a result of metabolic adjustment. Results from our laboratory (Johnson-Flanagan and Singh unpublished data) demonstrated that both plastid and nuclear rRNA levels increase in response to low temperature acclimation. Sarhan and Chevrier (1985) also reported that during the initial phase of low temperature acclimation, RNA polymerase I was more active compared to RNA polymerase II, again suggesting that rRNA was synthesized at a higher rate compared to mRNA.

Kurkela and Franck (1990) compared the predicted amino acid sequence of the KIN 1 protein (low temperature induced protein from Arabidopsis) with several group one antifreeze proteins (AFPs) from arctic flounder and found that the amount of identical amino acids between KIN 1 and a small AFP was 28%; the amount of biochemically similar amino acids was 41%. Both bn28 and another kin gene (Gilmour et al., 1992) code for polypeptides having significant homology with the kin 1 gene product. Therefore, we hypothesized that there may be a consensus sequence coding for the alanine-rich region in all genes coding for alanine-rich polypeptides. Northern blots probed with a synthesized oligonucleotide specific to the consensus sequence only detected a transcript in size similar to bn28 that appeared from one day of low temperature acclimation and remained throughout the 42 day experimental time, then disappeared within 12 hours of deacclimation. No expression was shown in nonacclimated leaves. This suggests that the alanine-rich polypeptides identified by *in vivo* labeling were encoded by several different genes containing coding sequences that were significantly different to the alanine-coding region of BN 28.

According to our calculation, the bn28 gene codes for a 6.5 kD

polypeptide. Thus, the 6.5 kD polypeptide detected in [^{3}H]-alanine *in vivo* labeling experiments was probably the product of gene bn28 (Figure 2) or another member of gene bn28 family. However, in the present study, the 6.5 kD alanine-rich polypeptide was detected on the first day of low temperature acclimation; While the BN28 polypeptide could not be detected among newly synthesized polypeptides in the earlier stage of acclimation even by immunoblots (Boothe et al., unpublished). Together, these results strongly suggest that this 6.5 kD alanine-rich polypeptide is another member of gene bn28 family.

In conclusion, during low temperature acclimation in Cascade, two classes of polypeptides were induced. One of these consisted low molecular weight alanine-rich polypeptides which may play an important role in adjusting plants growth at low temperature. There does not appear to be an alanine-rich region in these proteins that share homology with the antifreeze proteins. The second class of polypeptides was detected by methionine labeling and their appearance coincides with the onset of freezing tolerance. The exact role of these polypeptides is uncertain. The results from both *in vitro* translations and Northern blotting suggest expression of polypeptides induced by low temperature is regulated at the transcriptional level.

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